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Inhibition of Ruminal Cellulose Fermentation by Extracts of the Perennial Legume Cicer Milkvetch (*Astragalus cicer*)

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Cicer milkvetch (*Astragalus cicer* L.) is a perennial legume used as a pasture or rangeland plant for ruminants. A study was undertaken to determine whether reported variations in its ruminal digestibility may be related to the presence of an antinutritive material. In vitro fermentation of neutral detergent fiber (NDF) of cicer milkvetch by mixed rumen microflora was poorer than was the fermentation of NDF in alfalfa (*Medicago sativa* L.). Fermentation of cicer milkvetch NDF was improved by preextraction of the ground herbage with water for 3 h at 39°C. Such water extracts selectively inhibited in vitro fermentation of pure cellulose by mixed ruminal microflora and by pure cultures of the ruminal bacteria *Ruminococcus flavefaciens* FD-1 and *Fibrobacter succinogenes* S85. Inhibition of the cellulose fermentation by mixed ruminal microflora was dependent upon the concentration of cicer milkvetch extract and was overcome upon prolonged incubation. Pure cultures exposed to the extract did not recover from inhibition, even after long incubation times, unless the inhibitory agent was removed (viz., by dilution of inhibited cultures into fresh medium). The extract did not affect the fermentation of cellobiose by *R. flavefaciens* but did cause some inhibition of cellobiose fermentation by *F. succinogenes*. Moreover, the extracts did not inhibit hydrolysis of crystalline cellulose, carboxymethyl cellulose, or *p*-nitrophenylcellobioside by supernatants of these pure cultures of cellulolytic bacteria or by a commercial cellulase preparation from the fungus *Trichoderma reesei*. The agent caused cellulose-adherent cells to detach from cellulose fibers, suggesting that the agent may act, at least in part, by disrupting the glycocalyx necessary for adherence to, and rapid digestion of, cellulose.

Cicer milkvetch (chickpea milkvetch, *Astragalus cicer* L.) is an introduced perennial legume which has gained attention as a potential forage crop for the central and northern Great Plains and Pacific northwest regions of North America because of its low lignin content (6), acceptable yields (23), overwintering ability (22), leaf retention during environmental stress (13, 27), and resistance to insects (6). Although animal performance on cicer milkvetch is often good (17, 18), utilization of cicer milkvetch by ruminant livestock is sometimes problematic. While this forage does not cause bloat, it has been reported to occasionally cause photosensitization in lightly pigmented cattle (10), and there is some evidence that its fiber components are fermented less readily than are those of other forages (6). Such depression in fiber utilization may reflect purely structural factors (e.g., cell wall lignification) or the presence of toxic compounds at concentrations inhibitory to ruminal microflora. The purpose of this investigation was to examine whether the variable digestibility of cicer milkvetch by ruminal microorganisms is due to plant metabolites which inhibit fermentation of plant structural polysaccharides.

MATERIALS AND METHODS

Plant material. Cicer milkvetch (*A. cicer*, cultivars Monarch and Lutana) and alfalfa (*Medicago sativa* L., cultivar Apollo II) were grown at the Iowa State University Agronomy and Agricultural Engineering Research Center near Ames in 1984 and 1985. Details of cultivation and harvesting have been described previously (6). Monarch cicer milkvetch from the same batch of seeds was also grown in a greenhouse under supplemental lighting (14-h-day–10-h-night regime) during the summer and fall of 1991 at the U.S. Dairy Forage Research Center in Madison, Wis. Additional Monarch cicer milkvetch, grown near Fort Collins, Colo., was provided by C. E. Townsend. Cicer milkvetch genetic clones, developed from the Monarch cultivar and selected for high and low palatability to sheep, were generously provided by N. J. Ehlke, from plants grown in 1991 at the University of Minnesota Agricultural Experiment Station at Rosemount, Minn.

Extracts. Samples of air-dried forage (whole herbage, stems, or leaves) were Wiley milled through a 1-mm screen. Ground forage (1 to 5 g) was amended with 10 ml of water or McDougall buffer (12) per gram of forage and incubated on a rotary shaker at 39°C for 3 to 4 h. The resulting mixture was filtered through 40-μm Nitex nylon mesh (Tetko, Elmsford, N.Y.), with additional squeezing of filtration material to enhance recovery of entrapped liquid. Extracts were placed in serum vials, and their headspaces were evacuated and flushed with N_2 prior to their addition to fermentation vials. The residues retained in filters were dried in vacuo at 39°C prior to use in some fermentations; some residues were extensively rinsed with deionized water before drying.

In vitro fermentations. Fermentations were performed under oxygen-free CO_2 in 50-ml serum vials (15) containing 100 mg of Sigmacell 50 microcrystalline cellulose and 7.0 ml of McDougall buffer (supplemented with 10 mM NH_4Cl, 0.0002% [wt/vol] resazurin, and 0.025% [wt/vol] each of cysteine-HCl · H_2O and Na_2S · $9H_2O$). For some experiments, 250 mg of forage was substituted for the cellulose.

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Vials were warmed to 39°C, amended with extract (0.05 to 0.40 ml, as indicated), and inoculated with 2.4 ml of freshly collected and diluted rumen fluid (25) from a fistulated nonlactating Jersey cow maintained on an alfalfa hay diet. Control vials lacked cellulose or cicer milkvetch extract. Vials were incubated without shaking at 39°C, usually for 18 to 24 h. Triplicate or quadruplicate vials were run for each treatment condition.

Fermentations with the bacterial pure cultures *Ruminococcus flavefaciens* FD-1 and *Fibrobacter succinogenes* S85 were performed as described above, except that the medium used was a modified Dehority's medium (19; modified according to reference 25) with 0.1% (wt/vol) cysteine-HCl as a reducing agent. Fermentation vials were inoculated from cellulose-limited chemostat cultures operating at pH 6.3 and a dilution rate of 0.02 h^{-1} (26). Growth studies on cellobiose were conducted in anaerobic culture tubes (1) containing similar media lacking cellulose and inoculated from batch cultures grown 15 h on cellobiose as the energy source. For the cellobiose studies, growth was monitored turbidimetrically at 600 nm; zero-time A_{600} values varied from 0.07 to 0.08 in the absence of cicer milkvetch extract and from 0.14 to 0.23 in the presence of extract.

Recovery of substrate. At the end of the incubation period, cellulose was recovered by a neutral detergent fiber (NDF) procedure adapted for finely divided particulate cellulose (25). The residual dry weight of cellulose was corrected for NDF in inoculated controls lacking cellulose and for NDF introduced with the cicer milkvetch extract. For experiments with forages as growth substrates, NDF was recovered and quantitated by the method of Goering and Van Soest (4), except that residual NDF was collected on preweighed GF/D glass fiber filters (Whatman, Maidstone, United Kingdom). For all substrate recovery methods, decalin and Na sulfite were omitted from the neutral detergent solution, and triethylene glycol replaced 2-ethoxyethanol.

Electron microscopy. *F. succinogenes* S85 was grown in batch culture with Avicel PH101 as the cellulose source. After 20 h of incubation, cultures (3.0 ml) were exposed to 0, 0.1, or 1% (vol/vol) of cicer milkvetch extract and shaken for 10 min. The suspensions were treated with 1 ml of 8% glutaraldehyde for 30 min, and the entire sample was then gravity filtered through a GF/D glass fiber filter that had previously been trimmed to a 25-mm diameter and placed inside an Al seal closure (Wheaton, Millville, N.J.). After filtration was completed, a second filter and a plastic washer having a center diameter of 10 mm were placed over the first filter, and the entire "sandwich" was sealed with a hand crimping tool. The completed sandwich containing the entrapped cellulose fibers was successively dried through increasing concentrations of ethanol and then was critical-point dried under CO_2. The sandwich was then disassembled, and the original cellulose-containing filter was shadowed with Au by using a Technics Hummer VI sputter coater operated at 5 mA for 2 min. The coated fibers were mounted on Al stubs and examined with a JEOL JSM-35CF scanning electron microscope at an accelerating voltage of 8 kV.

Enzyme assays. Avicelase activity was determined by incubating 0.96 ml of supernatants of bacterial cultures (grown under different conditions) with 1.00 ml of acetate buffer (0.2 M, pH 5.0) and 10 mg of Avicel PH101 microcrystalline cellulose in 5-ml serum vials. After 16 h of incubation at 39 or 50°C and 200-rpm shaking, 1.5-ml samples were centrifuged at 12,000 × *g* for 5 min, and reducing sugars in the supernatants were assayed colorimetrically by using the dinitrosalicylic acid method of Miller et al. (14). Assays with soluble substrates were conducted in 1.7-ml tubes at 39°C. Carboxymethyl cellulase assay mixtures contained 0.50 ml of 2% (wt/vol) carboxymethyl cellulose in 0.10 M Na acetate buffer (pH 5.0), plus 0.50 ml of culture supernatant. After 4 to 8 h of incubation, the reaction was stopped by addition of 0.25 ml of 8% Na_2CO_3, and reducing sugars were measured as described above. Cellobiase activity was assayed by the method of Gardner et al. (3), using *p*-nitrophenyl-β-D-cellobioside as substrate. All assays were also conducted with *Trichoderma reesei* cellulase instead of the bacterial culture supernatants. For these assays, the enzyme was diluted in the above-mentioned buffer and used at a final concentration of 13 to 29 U per assay tube, and the incubation temperature was 50°C.

Chemicals. Sigmacell 50, cellulose CF-1, *p*-nitrophenyl-β-D-cellobioside, Na carboxymethyl cellulose, triethylaminoethyl cellulose, carboxymethyl cellulose, *T. reesei* cellulase, and potato phenol oxidase were obtained from Sigma (St. Louis, Mo.). Avicel PH101 was obtained from Fluka AG (Ronkonkoma, N.Y.). Darco G-60 activated carbon was obtained from Aldrich (Milwaukee, Wis.). Amorphous cellulose was prepared by chemical treatment of cellulose CF-1 by the method of Isogai and Atalla (5) or by ball-milling of CF-1 in a Spex mill (Spex Industries, Edison, N.J.) for a total of 4 h (eight 30-min cycles, with cooldown to room temperature between cycles).

Statistics. Statistical differences were determined by an analysis of variance with mean separations performed by Duncan's new multiple range test (21).

TABLE 1. Fermentation of NDF from forages by mixed rumen microflora before and after extraction with aqueous buffer

Forage	NDF content (% of dry matter)	% of NDF degraded at[a]:	
		16 h	48 h
Monarch cicer milkvetch[b]	29.5^x	5.1^x	15.5^x
Monarch cicer milkvetch residue[c]	42.8^y	7.5^x	44.0^y
Apollo II alfalfa[b]	42.0^y	21.2^y	39.7^y
Apollo II alfalfa residue[c]	53.1^z	20.3^y	40.6^y

[a] Mean values for triplicate cultures. Values in same column having different superscripts differ significantly ($P < 0.05$).
[b] Composited material from six cuts harvested in 1984 and 1985.
[c] Residue was prepared by collecting solids after extraction by McDougall buffer and drying in vacuum oven at 40°C prior to addition to fermentation vials.

RESULTS AND DISCUSSION

In vitro fermentation of water-extracted and unextracted cicer milkvetch NDF. In vitro fermentations of NDF in Monarch cicer milkvetch and Apollo II alfalfa were examined after 16- and 48-h incubations with mixed rumen microflora (Table 1). Less NDF was removed from the cicer milkvetch than from the alfalfa at both 16 and 48 h; however, the digestibility of the cicer milkvetch NDF was improved considerably if the ground forage was first extracted with warm McDougall buffer. In this case, the amount of NDF degraded from the cicer milkvetch solids following extraction was similar to that of alfalfa after 48 h of incubation, even though the cicer milkvetch residue was not exhaustively rinsed to remove inhibitory compounds prior to recovery and testing. Subsequent experiments revealed that water

TABLE 2. Fermentation of cellulose by mixed ruminal microflora treated with cicer milkvetch extracts derived from different plant parts

Cicer milkvetch material (cultivar)[a]	Source[b]	Cellulose (mg) degraded after 24 h at different extract concentrations[c]			
		Leaf extract		Stem extract	
		1%	3%	1%	3%
Monarch	G (immature)	44.3^{z}	31.1^{y}	39.7^{y}	47.4^{z}
	G (mature)	43.8^{z}	18.7^{z}	42.8^{y}	43.0^{y}
L2-17-34D	F	19.4^{x}	0.1^{x}	39.2^{y}	42.7^{y}
L7-18-G56	F	20.5^{x}	0^{x}	35.5^{y}	34.1^{y}
L14-11-F39	F	32.9^{y}	1.1^{x}	34.7^{y}	37.9^{y}
H6-2-65D	F	5.9^{w}	0.6^{x}	42.1^{y}	41.8^{y}
H14-7-65G	F	1.6^{w}	0.2^{x}	42.4^{y}	18.8^{x}
H16-1-66	F	9.1^{w}	0.8^{x}	42.8^{y}	41.5^{y}

[a] L and H, cultivars selected for low and high palatability, respectively, to sheep.

[b] G, greenhouse-grown in Madison, Wis., in 1991; leaf maturity was determined on the basis of plant material harvested on the same date. F, field-grown at Rosemount, Minn., in 1991.

[c] A total of 1 and 3% (vol/vol) of an extract was prepared as 1 g of dry plant material per 10 ml of water, equivalent to 1 and 3 g, respectively, of plant material per liter of culture volume. Values in the same column having different superscripts differ significantly ($P < 0.05$). The amount of cellulose degraded after 24 h in control cultures in the absence of extract was 38.1 mg (^{y}).

was as effective as McDougall buffer in removing the inhibitory agent. The data indicate that the inhibition of ruminal digestion of cicer milkvetch fiber is due to the presence of a water-soluble agent.

Effect of extracts on cellulose digestion by mixed ruminal microflora. Addition of the freshly prepared aqueous extracts of Monarch cicer milkvetch to in vitro fermentations of microcrystalline cellulose at concentrations of 3% (vol/vol) normally resulted in considerable, and sometimes total, inhibition in the amount of cellulose degraded by mixed ruminal microflora after 16 h; less inhibition was noted after 24 h, and inhibition was sometimes completely overcome by 48 h (data not shown). Inhibition was dose dependent, and transient inhibition of cellulose digestion was often observed at extract concentrations as low as 1% (vol/vol).

Parallel testing of extracts from six different Ames, Iowa, harvests (three cuts each in 1984 and 1985) revealed that only the first-harvest material from 1984 (i.e., material that had not been previously cut) lacked significant inhibitory activity. While the remaining harvests showed inhibitory activity, these differences generally were not significantly different from one another ($P > 0.05$). Parallel experiments with an extract of Lutana cicer milkvetch (grown in adjacent field plots) yielded similar levels of inhibition, while extracts from Apollo II alfalfa showed no inhibition of cellulose fermentation at 16 or 24 h of incubation. Somewhat stronger inhibition was noted with Monarch cicer milkvetch grown in 1991 near Fort Collins, Colo.

Testing of plant material grown in the field or greenhouse in 1991 indicated that the inhibitor was found in leaves but not stems (Table 2). Separation of the material by plant maturity revealed that the strongest inhibition was obtained with more-mature leaf tissue. Extracts prepared from genetic clones selected from the Monarch cultivar for high palatability gave greater inhibition than did those prepared from cultivars selected for low palatability (Table 2). The variation in inhibitory activity observed in different cicer milkvetch samples suggests that the varying levels of the agent resulting from different genetic backgrounds or different growth environments may explain conflicting data on the digestibility of this forage (6, 10, 13, 23). Most extracts caused complete inhibition of in vitro cellulose digestion at concentrations of 3% (vol/vol). At an assumed bovine rumen liquid volume of 50 liters, this translates to a mere 0.15 kg of dry cicer milkvetch to completely (but transiently) inhibit the ruminal cellulose fermentation. The fact that greater inhibitory activity was extracted from older leaves suggests that the inhibitor may be a secondary metabolite produced late in development or in response to stress (e.g., cutting). The inhibitor was also present in fresh leaves, but dried material was used for the experiments described here owing to its ease of being ground to prepare a substrate suitable for water extraction.

Effect of extract on individual species of ruminal cellulolytic bacteria. Extracts of Monarch cicer milkvetch at a 4% (vol/vol) concentration caused complete inhibition of the cellulose fermentation by pure culture of either *R. flavefaciens* FD-1 or *F. succinogenes* S85. In this experiment, the amount of cellulose digested after 18 h of incubation averaged 35.5 ± 0.1 mg (FD-1) and 35.4 ± 0.1 mg (S85) in the absence of extract and <0.1 mg in its presence. Unlike the mixed ruminal microflora cultures, prolonged incubation of other vials inoculated at the same time revealed that this inhibition was not overcome even after 7 days of incubation. In contrast to the inhibitory effects of the cicer milkvetch extract on cellulose digestion, fermentative growth on cellobiose (β-1,4-D-glucosyl-D-glucopyranose, the basic stereochemical repeating unit of cellulose) by *R. flavefaciens* FD-1 was not inhibited by the extract: (A_{600} increased after 18 h by 0.628 ± 0.013 and 0.647 ± 0.006 in the absence or presence, respectively, of 4% (vol/vol) extract. Cicer milkvetch extracts did not inhibit fermentative growth of *F. succinogenes* S85 on cellobiose at early time points but did cause some decrease in the ultimate amount of growth observed: A_{600} over the 18-h incubation increased by 1.07 ± 0.04 and 0.627 ± 0.050 in the absence and presence, respectively, of 4% (vol/vol) of extract.

In other experiments, cicer milkvetch extract was shown to completely inhibit fermentation of all types of cellulose tested, including two crystalline and two amorphous celluloses.

The inhibitory agent did not appear to be directly cytotoxic, since cultures of *R. flavefaciens* exposed to cicer milkvetch extract (and thus not growing) for as long as 24 h resumed cellulose fermentation and growth upon dilution into fresh medium containing cellulose but lacking the extract. Lag times before regrowth (estimated visually) were similar to those of parallel cultures that had been starved by inoculation into fresh media lacking both cellulose and extract prior to reinoculation into fresh media. Regrown, extract-inhibited cultures retained sensitivity to further exposure to the cicer milkvetch extract, indicating that they were rescued survivors rather than resistant mutants. Survival could not be quantitated, however, since the vast majority of cells were attached to cellulose particles and thus were not countable either microscopically or by dilution plating.

Microscopic examination of crystal violet-stained culture wet mounts revealed that cellulose-containing media inoculated with both culture and cicer milkvetch extract did not show a significant increase in cell numbers over control cultures lacking both cicer milkvetch extract and cellulose.

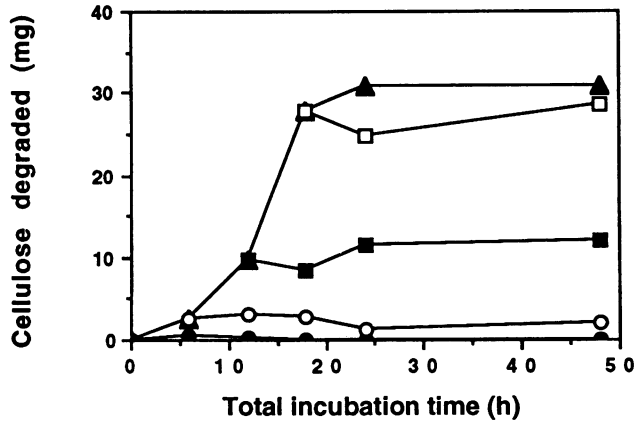

FIG. 1. Effect of time of addition of cicer milkvetch extract on the degradation of cellulose by *R. flavefaciens* FD-1. Extract concentration was 3% (vol/vol) of a slurry prepared as described in Materials and Methods. Extract was prepared from ground, dried whole herbage grown near Fort Collins, Colo., in 1991. Extract was added at 0 (●), 6 (○), 12 (■), or 18 (□) h or not added at all (▲).

Few of the cells in the inhibited cultures adhered to the cellulose particles. When batch cultures of *R. flavefaciens* FD-1 were treated with extract, immediate cessation of cellulose digestion occurred regardless of its time of addition (Fig. 1). Growing cultures of *R. flavefaciens* FD-1 that were well attached to cellulose fibers did not exhibit obvious detachment from the fibers upon exposure to cicer milkvetch extract for brief periods of time (<1 h), although prolonged exposure resulted in some detachment of cells. By contrast, treatment of cellulose-grown *F. succinogenes* S85 with cicer milkvetch extract caused a rapid detachment of cells from the cellulose fibers (Fig. 2) The greater detachment observed with *F. succinogenes* than with *R. flavefaciens* is in accord with known differences in the thickness and tenacity of the glycocalyces of these species (8) and parallels the more complete removal of *F. succinogenes* upon treatment with methylcellulose, a synthetic cellulose analog (7). The data suggest that the agent in cicer milkvetch may act by disrupting the glycocalyx necessary for adherence of cells to cellulose, even though (in the case of *R. flavefaciens*) this disruption may not necessarily proceed to the point of complete detachment of cells from the cellulose.

Effect of extract on microbial cellulase activities. The extract did not inhibit the hydrolysis of Avicel, Na carboxymethyl cellulose, or *p*-nitrophenyl-β-D-cellobioside by cell-free supernatants of *F. succinogenes* or *R. flavefaciens* cultures or by the cellulase complex of the aerobic fungus *T. reesei*. In these hydrolysis experiments, supernatants from vials amended with cicer milkvetch extract contained slightly more reducing sugars than did controls lacking the extract; however, parallel testing of controls lacking enzyme or substrate revealed that this excess was due to background levels of reducing sugars in the cicer milkvetch extract. The data indicate that the cicer milkvetch agent does not directly inhibit enzymatic hydrolysis of cellulose by cell-free cellulases.

Properties of the inhibitory agent. Inhibitory activity was retained in extracts subjected to boiling (10 min) or to treatment with cellulose, anion exchange resins (triethylaminoethyl cellulose or Dowex AG1-X8), cation exchange resins (carboxymethyl cellulose or Dowex AG50W-X8), diethyl ether, or phenol oxidase. The inhibitor was retained by an ultrafiltration membrane having a nominal molecular mass cutoff of 10 kDa but was only partially retained by a 30-kDa membrane. Inhibitory activity was removed by treatment

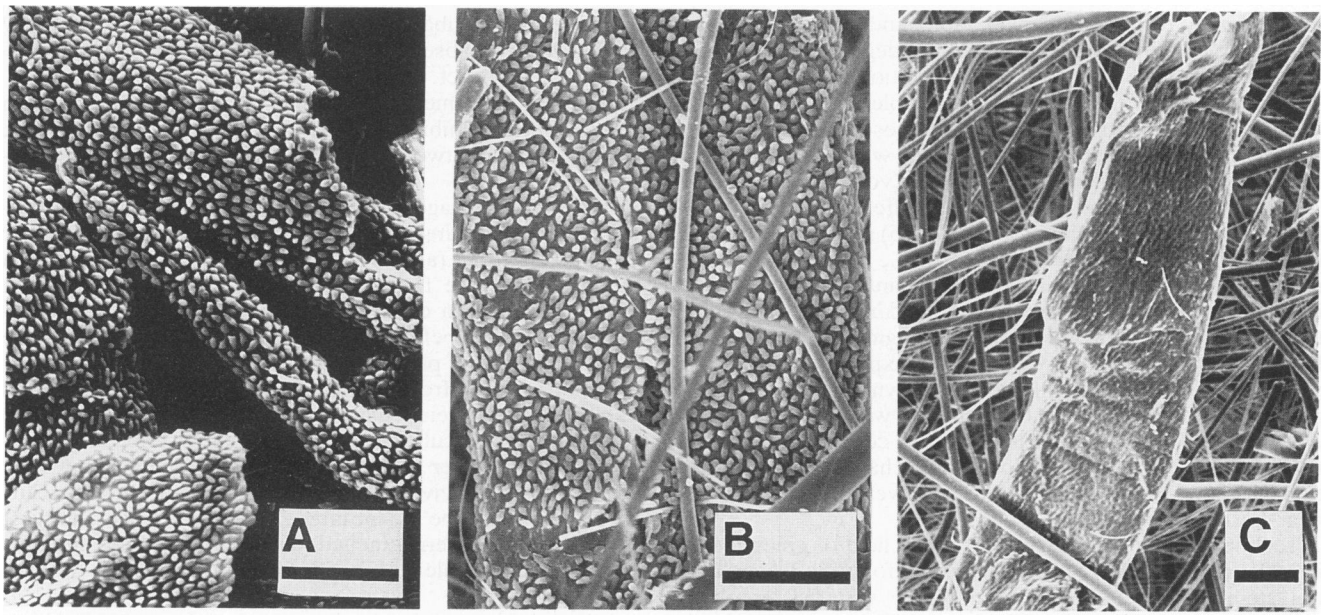

FIG. 2. Scanning electron photomicrographs showing the detachment of cellulose-adherent cultures of *F. succinogenes* S85 by cicer milkvetch extract. After 20 h of growth of bacterial cultures in cellulose-containing media, extracts were added to final concentrations of 0% (A), 0.1% (B), or 1.0% (C) (vol/vol) (see Materials and Methods for details of extract preparation). The cultures were then shaken 10 min prior to fixation and preparation for electron microscopy. (In panels B and C, the glass fiber filters used to recover the larger cellulose particles are readily observable). For all panels, bar = 10 μm.

with activated carbon (Darco G-60) or by freezing and subsequent thawing of the crude extract.

Relationship of the inhibitory agent to other antinutritive compounds. Several plant products have been previously reported to inhibit ruminal cellulose fermentation in vitro. The inhibitory effects of forage phenolics on cellulases have been widely reported (for a summary, see reference 11), and extracts of lespedeza (*Sericea cuneata*) inhibit cellulose digestion by mixed ruminal microflora in vitro, apparently because of the presence of condensed tannins (20), which are known to bind extracellular enzymes (29). However, cicer milkvetch reportedly contains only low levels of tannins (2). In addition, our results indicate that the agent did not lose activity upon treatment with phenol oxidase, and partial purification of the agent reveals that it does not have the UV-visible absorption characteristics of phenolics or tannins (24). Similarly, the inhibitor does not appear to be a nitrotoxin or cyanogenic glucoside, because of the reported absence of these materials in *A. cicer* (28) and the apparent high molecular mass (>10 kDa) of the active fraction of the extract. Purification of the agent, currently in progress, will provide an interesting and useful tool for further examination of the ruminal cellulolytic process, particularly the role and mechanism of bacterial attachment to cellulose. Currently available inhibitors specific to the cellulolytic process are exclusively synthetic compounds such as epoxyalkyloligosaccharides, which inhibit some cellulolytic enzymes (9) and methylcellulose, which can both detach ruminal bacteria from cellulose and inhibit cellulolytic enzymes (16).

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